Please replace the specification as follows:

IN THE SPECIFICATION

Please replace the paragraph on page 44, ln. 18 through page 45, ln. 5 with the following:

Bacterial Strains and Plasmids. *Streptococcus pneumoniae* serotype 14 (ATCC, 10801 University Boulevard, Manassas, VA 20110-2209) was used in this study for isolation of genomic DNA. *E. coli* strain DH5 (Life Technologies, Gaithersburg, MD) was used for initial cloning and production of plasmid DNA. *E. coli* strain BL21 (DE3)ompA, used for protein expression, was derived from BL21 (BE3) (Novagen) (see U.S. Patent No. 5,439,808 for details). *S. pneumoniae* was grown overnight in Todd-Hewitt (TH) broth at 37C without shaking under 7.5% CO_2. *E. coli* strains were grown in Luria-Bertani (LB) broth, supplemented with carbenicillin (50-100 µg/ml) or kanamycin (50 µg/ml) as needed. The plasmid vectors pUC-19 and/or pBluescript II SK+ (Stratagene) were used for cloning fragments to be sequenced and the plasmids pET-17b and pET-24a (Novagen) were used for cloning fragments to be expressed.

Please replace the paragraph on page 51, ln. 23 through page 52, ln. 20 with the following:

Clones containing soluble pneumolysin are selected for the next step in the screening procedure, which consists of discarding the supernatant by aspiration, washing the pellet with TEN buffer twice, and solubilizing the pellet in 5 ml of 8 M urea prepared in TEN buffer. After sonicating for 2 min, the urea solution is quickly centrifuged to remove aggregates and added dropwise to 45 ml of refolding solution, under constant stirring at 4 °C. The refolding solution is then loaded onto a 2 ml DEAE-SEPHAROSE-FF column, pre-equilibrated in Buffer A (25 mM Tris.HCl, pH 8.0). The column

is washed with Buffer A and the bound protein is eluted with a gradient of 0 to 1 M NaCl. The properly refolded pneumolysin mutant should elute as a single peak between 13 and 20% Buffer B (25 mM Tris.HCl, 1 M NaCl, pH 8.0) similarly to what is observed for the wild-type. The protein peak is further analyzed by HPLC on a SUPEROSE 12 column and both elution time, aggregate/monomer ratio, and hemolytic activity are evaluated (see Table 4). The selected mutant(s) should present a single monomeric species with a Stokes radius comparable to the wild-type. Five clones (pNVJ1, pNVJ20, pNVJ22, pNVJ45, pNVJ56) with high yields of monomeric modified polypeptides were selected for further analysis including nucleic acid sequencing. The amino and nucleic acid substitutions of these clones are shown in Tables 5A and 6. Throughout the specification and claims, proteins are given the name of the vector that encodes them.

Please replace the paragraph on page 59, lns. 2-28 with the following:

Pneumolysin expressed in *E. coli* cells harboring the expression vector pNV19 was isolated from inclusion bodies by resuspending and lysing the cells in TEN buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA pH 8.0), with an air driven cell disrupter (Stansted Fluid Power Ltd.) under a pressure of 8,000 psi. The cell lysate was centrifuged at 13,000 rpm at 4C for 20 minutes; both pellet and supernatant were saved for isolation of soluble and aggregated pneumolysin, respectively. The inclusion bodies were washed three times with TEN buffer and stored at -70C Purification and subsequent refolding were achieved by solubilizing the inclusion bodies in an 8 M urea solution (freshly prepared in TEN buffer), followed by PEG-assisted refolding. Polypeptide solutions in 8 M urea (200 µg/ml) were diluted 10-fold by drop-wise addition to a refolding solution, consisting of 20 µM of PEG 8,000 in 25 mM Tris-HCl, pH 8.0, under constant stirring at 4C. The sample was clarified and loaded into a DEAE- SEPHAROSE Fast Flow ion exchange column (Pharmacia) equilibrated in 25mM Tris-HCl, pH 8.0. A gradient of 0-1 M

NaCl was applied and pneumolysin containing fractions were identified by detection of hemolytic activity, as described below, and by SDS-PAGE. The purified fractions were concentrated by using an Amicon concentrator and PM30 membrane. Aliquots of purified polypeptide were tested for hemolytic activity, and analyzed by SDS-PAGE and size exclusion chromatography, using a SUPEROSE 12 column.

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Please replace the paragraph on page 63, lns. 5-24 with the following:

PnC type 14 polysaccharide (ATCC Lot #2016107) (390 mg) was dissolved in 16 ml of 0.5 N NaOH, and the solution was heated at 70C for 3 hours. Following cooling of the solution, 1.93 ml of glacial acetic acid was added to bring the pH to 4. After addition of 3 ml of 5% (w/v) $NaNO_2$, the reaction mixture was kept stirring at 4C for 2 hours. The sample was then diluted to 50 ml with deionized water and the pH was adjusted to 7 with 0.5 N NaOH. Excess reagents were dialyzed out by diafiltration with DI water through a SPECTRA/POR molecular porous membrane tubing (MWCOL:3,500), and the retentates freeze-dried. The deaminated type 14 polysaccharide was then molecular sieved on a SUPERDEX G-200 (Pharmacia) column using PBS as eluent. Fractions eluting from the column with molecular weight between 5000 and 15,000 as determined by Chromatography/Multiangle Laser Light Scattering using a SUPEROSE 12 column (Pharmacia) were pooled and dialyzed against DI water through a SPECTRA/POR molecular porous membrane tubing (MWCOL 3,500) and freeze-dried.

G10

Please replace the paragraphs on page 64, ln. 3 through page 65, ln.3 with the following:

Modified pneumolysin polypeptides in 0.2 M phosphate buffer (pH 8) at a concentration of 5 mg/ml were mixed with 2.5 equivalents (by weight) of PnC 14 polysaccharide-fragment together with 2 equivalents (by weight) of

G11

recrystallized sodium cyanoborohydride . Reaction mixtures were incubated at 37°C for 24 hours. Conjugates were then purified from the free components by passage through a SUPERDEX G200 (Pharmacia) column using PBS containing 0.01% thimerosal as an eluent. Fractions eluting from the column were monitored on a Waters R403 differential refractometer and by UV spectroscopy at 280 nm. The fractions containing the conjugates were pooled, sterile-filtered through a 0.22 μm Millipore membrane and then stored at 4C. Polypeptide and carbohydrate content were measured by the methods of Bradford and Dubois respectively. Polysaccharide content in the resulting conjugates were approximately 30%.

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Tetanus toxoid conjugates for use as control, were also produced as described above and as follows: Tetanus toxoid (Serum Statens Institute) was first passed through a molecular sieve column (SUPERDEX G-200 Pharmacia) in order to obtain the monomer form of the toxoid. For conjugation, 12 mg of the monomer and 36 mg of the PnC 14 polysaccharide-fragments were dissolved in 600 μl of 0.2 M phosphate buffer pH 7.2. Recrystallized sodium cyanoborohydride (24 mg) was then added to the solution which was then incubated at 37°C for 3-days. The conjugate was purified as above. The conjugates had polysaccharide contents in the 25-30% range (see Table 10).

Please replace the paragraphs on page 66, lns. 4-28 with the following:

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Micro titer plates (Nunc Polysorb ELISA plates) were sensitized by adding 100 μl of type 14 polysaccharide-fragment (MW ca: 10,000)/HSA conjugate (2.5 μg/ml) in PBS. The plates were sealed and incubated at 37C for 1 hour. The plates were washed with PBS containing 0.05% TWEEN 20 (PBS-T) and blocked with 0.5% (w/v) BSA in PBS for 1 hour at room temperature. The wells were then filled with 100 μl of serial two-fold dilutions in PBS-T plates, 100 μl of peroxidase labeled goat anti-mouse IgG (H+L) (Kirkegaard and Perry Laboratories), and then washed five times with

PBS-T. Finally, 50 µl of TMB peroxidase substrate (Kirkegaard and Perry Laboratories) were added to each well, and following incubation of the plates for 10 minutes at room temperature, the reaction was stopped by the addition of 50 ul of 1 M H_3PO_4. The plates were read at 450 nm with a Molecular Device Amex microplate reader using 650 nm as a reference wavelength.

C12

Inhibition ELISA assay.

Microtiter plates (NUNC Polysorp) were coated with PLY (20 ng in 100 mL to each well) in PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7.4) for one hour at 37°C. After washing the plates with PBS + 0.05% TWEEN 20 (PBST), the plates were post-coated with 150 mL of PBS + 0.1% BSA, rewashed, and stored at 4°C until used.

Please replace the paragraph on page 67, lns. 1-21 with the following:

C13

Hyperimmune rabbit anti-PLY was diluted in PBST, added to the PLY coated plates, and incubated at room temperature for 1 h. After washing, 100 mL of goat anti-rabbit Ig-HRP conjugate (KPL) diluted in PBST according to the manufacturer's instructions were added to each well. The plate was incubated at room temperature for one hour and then washed again. 100mL of TMB microwell substrate (KPL) were added to each well. The reaction was stopped after 10 minutes by the addition of TMB one-component stop solution (KPL) and the $OD_{450\ nm}$ was immediately read. The dilution corresponding to 1/2 the maximum signal was chosen for the inhibition study. PLYD mutants as well as PLY as a control were diluted serially in three-fold ingrements in PBST containing the rabbit antiserum diluted such that the final mixture contained the dilution which gave half-maximal activity and applied immediately to the coated microtiter plates in duplicate. The plates were incubated at room temperature for one hour and processed. Inhibition was

G13 determined as percent of maximum signal achieved with dilute antiserum in the absence of any inhibitor.

Please replace the paragraph on page 71, lns. 6-24 with the following:

The oxidized PSs were separately coupled to recombinant pneumolysoid mutant 207 in which amino acid Phe residue 195 was replaced by Ile. In brief, the oxidized PSs and the protein (5 mg/ml) in 0.2 M sodium phosphate buffer were combined at a PS/protein ratio of about 2.5:1 by weight at room temperature and sodium cyanoborohydride (2 equivalents by weight) was then added. The conjugation mixtures were incubated at 37C for 2 days. After reduction of the residual aldehydes of the conjugated PS, with excess $NaBH_4$, the conjugates were purified from the reaction mixtures by passage through a column of SUPERDEX 200 PG (Pharmacia) eluted with PBS containing 0.01% thimerosal as the preservative, except for the type 23 conjugate where the conjugate was loaded onto a Q SEPHAROSE Fast Flow column, and eluted with 10 mM Tris-HCl, pH 7.5 using a gradient of 0.5 M NaCl. Fractions corresponding to the conjugates were pooled and analyzed for protein and carbohydrate content as described in example 8 (see Table 12).

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REMARKS

Applicants respectfully request favorable reconsideration in view of the herewith presented amendments and remarks.

Claims 35-37, 42-51, 53, and 60-79 are pending in this application. Claims 43-51 are allowed. Claims 35-37, 42, 53, and 60-79 are rejected. Please add additional claim 80.

Specification - Informalities

8) The specification has been objected to for the following reasons:

(a) Effective March 23, 1998, the address of the ATCC has changed. The specification has been amended to reflect this change and specifically to read: 10801